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Biocompatibility assessment of fibrous nanomaterials in mammalian embryos

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Abstract

Currently there is a growing interest in the use of nanotechnology in reproductive medicine and reproductive biology. However, their toxic effects on mammalian embryos remain poorly understood. In this work, we evaluate the biocompatibility of two fibrous nanomaterials (NMs): cotton cellulose nanofibers (CNF) and carboxylated multiwalled carbon nanotubes (MWCNT-COOH), by performing an investigation of the embryonic development, gene expression (biomarkers focused on cell stress, apoptosis and totipotency) and *in situ* apoptosis in bovine embryos. Exposure to NMs did not interfere in preimplantation development or in the incidence of apoptosis in the bovine embryo, but they did affect the gene expression. The results presented are important for an understanding of the toxicity of cotton CNF and MWCNT-COOH on mammalian embryos. To our knowledge, we report the first evaluation of biocompatibility between these NMs on preimplantation embryos, which may open a new window for reproductive biomedical applications.

Key words: Fibrous nanomaterials; Biocompatibility; Gene expression; Apoptosis

Nanomaterials (NMs) have garnered increasing interest recently in medicine and biology fields. Among existing NMs, cellulose nanofibers (CNF) and carbon nanotube (CNT) are fibrous NM which have received considerable attention. CNFs have emerged as attractive NMs due to their hydrophilicity, flexibility, mechanical strength, broad chemical-modifying capacity, biodegradability aspect and low cost.[1,2] CNTs have unique characteristics, such as large contact surface, stability, flexibility, stiffness, strength, as well as thermal and electrical conductivities.[3] These NMs can be applied in drug delivery,[4,5] regenerative medicine,[6,7] and diagnostic systems.[8]

In recent years, nanotechnology has been introduced into the fields of reproductive medicine and reproductive biology. Emerging reproductive applications of NMs include treatment of chronic disease, support of assisted reproduction techniques and embryogenesis.[9–11]

Despite their tremendous potential, little is known about the effects of these NMs on mammalian embryos. Further, most of the studies on NMs have focused on their applications in nanotechnology, often overlooking, during their design and fabrication, the toxic effects associated with their use. The unique chemical and physical properties of NMs, such as the small size, shape, and high reactivity, which enable their applications in diverse areas might also render them potentially toxic to cells and tissues.[12] The results found in the literature often show discrepancies and variability depending on the cell type under investigation, surface functionalization, and NM size. Therefore, in-depth studies are needed to better understand the potential deleterious effects of NMs and to optimize the use of nanotechnology in the field of biology and medicine.

In the present study, we used bovine embryos as the experimental model due to the ethical and practical work limitations when it comes to working with human embryos and,

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in many respects, bovine embryos constitute a good substitute model for human embryos. Moreover, bovine animals are normally monovulators just like humans, the embryos are about the same diameter and have a broadly similar pattern of energy metabolism, measured as oxygen, pyruvate, and glucose consumption and lactate production.[13] Approximately the same proportion of *in vitro*-produced bovine zygotes reach the blastocyst stage when *in vitro* and major zygotic genome activation is initiated at closely related stages.[14]

Thus, the aim of the present study was to evaluate *in vitro* biocompatibility of cotton CNF and carboxylated multiwalled carbon nanotubes (MWCNT-COOH), by investigating the developmental competence, gene expression of biomarkers related to cell stress and apoptosis (*PRDX1*, *BAX*, *HSP70.1*) and totipotency (*HAD1*, *OCT4*) and apoptosis in bovine embryos.

This work provides a direct comparison of the impact of cotton CNF and MWCNT-COOH on mammalian embryos. To our knowledge, we report the first *in vitro* assessment of the biocompatibility between these NMs and preimplantation embryos.

Methods

All chemicals were from Sigma Chemical (St. Louis, MO, USA), unless otherwise stated.

Preparation of nanomaterials

CNF was prepared by acid hydrolysis of commercial cotton fibers purchased from the local market. The fibers were finely chopped in a knife mill, passed through a 10-mesh sieve, dewaxed with 1:1 (v/v) ethanol:cyclohexane for 12 h in a soxhlet apparatus and then vigorously washed with tap water. The dewaxed samples were dried for 12 h at 100 °C in an air-circulating oven. About 5 g of fibers were dispersed in 100 mL of 6.5 M sulfuric acid at 45 °C and stirred vigorously for 75 min. After that, 500 mL of cold distilled water was added to stop the reaction. The sulfuric acid was partially removed from the resulting suspension by centrifugation at 8,000 × g for 15 min. The non-reactive sulfate groups were removed by centrifugation followed by dialysis. Then the fibers were resuspended and dialyzed against tap water with a tubing cellulose membrane (76 mm, D9402- Sigma) until the pH reached 6–7. The resulting suspension was sonicated (Branson 450 sonifier, Branson Ultrasonics, Danbury, USA) for 5 min (in ice bath) and stored in a refrigerator.

The MWCNTs were synthesized using a floating catalytic chemical vapor deposition process using ferrocene and ethylene as the transition metal and carbon precursors, respectively. After the synthesis, the MWCNTs were submitted to a simple purification process by washing and filtering several times with isopropyl alcohol in a Millipore filtration system in order to remove any non-reacted ferrocene and other carbon impurities. After the cleaning process, the MWCNTs were dried at 80 °C for 12 h and functionalized with carboxyl through oxidation in nitric/sulfuric acid for 15 min. The MWCNT-COOH were then washed in neutral pH, and dried at 60 °C during 12 h.

Transmission electron microscope (TEM) analysis

An aliquot of cotton CNF suspension was diluted and sonicated for 5 min. A drop of this resultant diluted suspension was deposited on a carbon micro grid net (400 meshes) and the grid was stained with a 1.5% solution of uranyl acetate and dried at room temperature. Samples MWCNT-COOHs were prepared by ultrasonic dispersing in ethanol and dropping on a carbon-coated copper grid. The NMs were characterized by TEM using an FEI Tecnai G2 Spirit electron microscope at 120 kV.

Zeta potential

The Zeta potential of the cotton CNF and MWCNT-COOH were determined by microelectrophoresis laser Doppler technique (Zetasizer Nano ZN; Malvern Instruments Ltd, Malvern, Worcestershire, UK).

In vitro production embryos

The oocytes were obtained from ovaries collected from slaughtered cows. After selection, oocytes were matured *in vitro* in TCM-199 media (Gibco Life Technologies, Inc., Grand Island, NY, USA), supplemented with 10% fetal calf serum (FCS; Nutricell, Campinas, SP, Brazil), 20 μg mL^{-1} follicle stimulating hormone (FSH; Pluset, Serono, Italy) and incubated at 5% CO_2, 38 °C in the air and 95% humidity for 24 h. *In vitro* fertilization was performed in 100-mL drops of Fert-TALP supplemented with 2×10^6 spermatozoa mL^{-1}, 20 mg mL^{-1} heparin and 6 mg mL^{-1} fatty acid-free bovine serum albumin (BSA) Fraction V and covered with mineral oil for 21 h at 38 °C under 5% CO_2 in humidified air. Presumptive zygotes were cultured in Charles Rosenkrans 2 (CR2aa) medium with 10% FCS at 5% CO_2, 38 °C and 95% humidity for 7 days.

Exposure of embryos to nanomaterials

Cotton CNF or MWCNT-COOH were dispersed in 2 μg mL^{-1} in CR2aa medium and treated with ultrasonic agitation under 200 W of power, at 24 kHz working frequency and 50% pulse factors per second (UP200, Hieslcher-Germany) for 1 min at 4 °C. Afterwards, cotton CNF or MWCNTs were diluted in CR2aa medium (final concentration of 0.2 μg mL^{-1}) and 10% FCS; they were subsequently used for embryo culture. The selection of this concentration was based on previous studies that we evaluated the cytotoxicity of cotton CNF on bovine fibroblast cells.[15]

On day 7 post-fertilization embryos at the blastocyst stage were randomly distributed into three culture groups: the control group (without nanomaterials; number of embryos = 43), the cotton CNF group (0.2 μg mL^{-1}; number of embryos = 41) and the MWCNT-COOH group (0.2 μg mL^{-1}; number of embryos = 46). Embryos in all groups were cultured in CR2aa medium, supplemented with 10% FCS and granulosa cell monolayer for 72 h in microdrops covered with mineral oil under 5% CO_2 at 38 °C in the air and 95% humidity. After 72 h of exposure to NMs during *in vitro* culture, the embryos at blastocyst stage (10 days post-fertilization) were recovered and submitted to apoptosis and gene expression analysis.

Table 1
Primer sequences used for relative gene expression analysis by real time polymerase chain reaction.

Gene symbol	Primer sequence (5′–3′)	Annealing temperature (°C)	Fragment size (bp)	GenBank accession number
HSP70.1	F – AACAAGATCACCATCACCAACG R – TCCTTCTCCGCCAAGGTGTTG	59	275	NM_174550
PRDX1	F – TGCCAGATGGTCAGTTCAAG R – CCTTGTTTCTTGGGTGTGTTG	53	224	NM_174431.1
BAX	F – TTGCTTCAGGGTTTCATCCAGGA R – CAGCTGCGATCATCCTCTGCAG	64	174	NM_173894
HAD1	F – GCGGGCGCAAGAACTCTTCCAA R – AGAGTTTGGGAGGGACGGGTTG	57	372	NM_001037444.1
OCT4	F – CCCACCCTGCAGCAAATTA R – GCTTGATCGTTTGCCCTTCT	50	105	NM_174580.2
ACTB	F – GACATCCGCAAGGACCTCTA R – ACATCTGCTGGAAGGTGGAC	53	205	NM_173979

Microscopic embryo development evaluation

After culture, hatching and degeneration rates of embryos were evaluated under a stereoscopic microscope (Nikon SMZ-745, Melville, USA).

Quantitative real-time PCR

Total RNA was extracted from three pools of ten blastocysts per treatment using the Rneasy Micro Kit (Qiagen GmbH, Hilden, Germany). The RNA samples were reversely transcribed using the SuperScript III First-Strand Synthesis Supermix (Invitrogen, Carlsbad, CA, USA), using oligo (dT) $_{20}$ primers, dNTP mix, Superscript™ III RT, RNaseOUT™, $MgCl_2$, RT buffer in a final volume of 20 μL. The gene expression was performed in triplicate using Real Time PCR (ABI Prism 7300 Sequence Detection Systems, Applied Biosystem, Foster City, CA, USA). The amount of cDNA used in the reactions ranged according to the optimal concentration previously identified. For amplification, 600 ng cDNA was used per reaction for heat shock protein 70.1 (*HSP70.1*), peroxiredoxin 1 (*PRDX1*) and BCL-2 associated X protein (*BAX*) genes, 400 ng cDNA for histone deacetylase 1 (*HAD1*) and octamer-binding transcription factor 4 (*OCT4*) genes, 200 ng cDNA for beta-ACTIN (*ACTB*). cDNA template was denatured at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, gene-specific primer annealing temperature for 30 s (Table 1), and elongation at 60 °C for 30 s. The relative quantification was made using the ΔΔCt method using the *ACTB* housekeeping gene expression.

Agarose gel electrophoresis

Agarose gels containing 1.5 μL mL^{-1} of SYBR® Safe DNA Gel Stain (Invitrogen) were prepared by using 20 mg mL^{-1} agarose in tris–borate–EDTA (TBE) buffer. DNA samples were mixed with the Gel Loading Dye Orange (New England Biolabs, Ipswich, MA) and loaded into the wells alongside a 50 pb DNA Ladder (Life Technologies, Inc., Grand Island, NY, USA). Electrophoresis was performed at 105 V and bands were visualized using a UV transilluminator (Eagle eye II, Stratagene, CA, USA).

Apoptosis analysis

Embryos at the blastocyst stage (on day 10 post-fertilization) from different groups were submitted to *terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end-labelling* (TUNEL) staining using a commercially available kit (Dead End Fluorimetric TUNEL System, Promega, Madison, WI, USA) according to manufacturer's instructions. Briefly, embryos were fixed in 4% paraformaldehyde at 4 °C and then permeabilized with 0.2% Triton X-100 (Promega, Madison, WI, USA), both in PBS (Nutricell). Positive control embryos were previously treated with DNase (Promega). After permeabilization, positive control and target samples were incubated in 100 μl drops with reagent mix containing enzyme solution (terminal deoxynucleotide transferase enzyme) and 90% staining solution (dUTP fluorescein conjugate) for 1 h at 37 °C in a dark humid chamber. Negative control embryos were incubated only in the staining solution without enzyme solution. After that, embryos were stained with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) plus 4′6-diamidino-2-phenylindole (DAPI) and mounted on slides for evaluation by fluorescence microscopy.

Total cell number and apoptotic cell number per embryo were counted and apoptotic cell index was calculated as the proportion of apoptotic cell/total cell number.

Statistical analysis

Data for the hatching and degeneration rates at 72 h after NMs exposition were analyzed using a chi-square test. Data of total cell number, apoptotic cell number and apoptotic index were analyzed by ANOVA and differences among means were compared by the Student–Newman–Keuls' (SNK) test using the general linear model (GLM) of SAS version 9.1 (SAS Institute, Cary, NC, USA). The data corresponding to the relative gene expression were analyzed using the pair wise fixed reallocation randomization test performed by REST® software.[16] $P < 0.05$ was considered significant. All data are presented as mean ± SEM.

Results

Characterizations of nanomaterials

The morphological study of the cotton CNF and MWCNT-COOH suspensions by TEM are shown in Figure 1. The images revealed that cotton CNF used in this study are elongate needle-like NMs with a diameter of about 6–18 nm and a length of 85–225 μm (Figure 1, *A*). From the TEM image

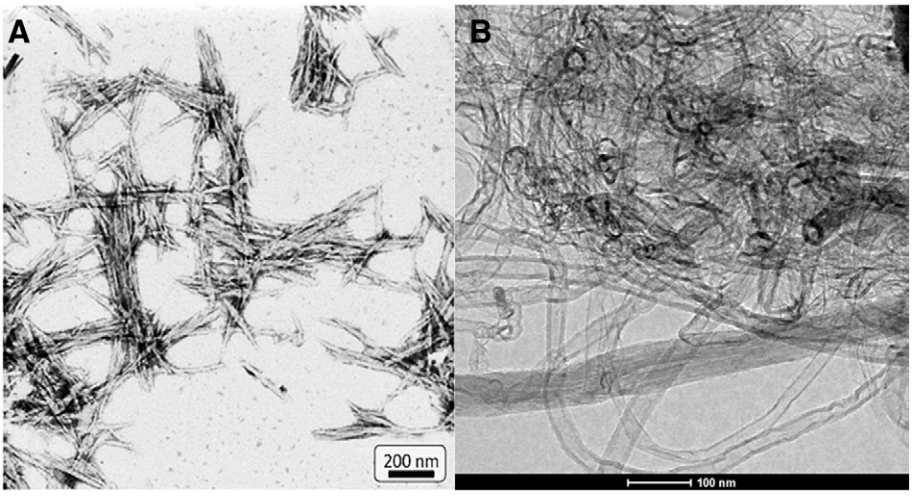

Figure 1. Transmission electron microscope (TEM) images of the cotton cellulose nanofibers (CNF) **(A)** and carboxylated multiwalled carbon nanotubes (MWCNT-COOH) **(B)**.

Table 2
Hatching and degeneration rate in bovine embryos cultured without nanomaterials, with 0.2 μg mL^{-1} cotton cellulose nanofibers (CNF) or multiwalled carbon nanotubes (MWCNT-COOH).

Exposure period	*n*	Group	Hatching (%)	Degeneration (%)
72 h	43	Control	60.76	5.15
	41	Cotton CNF	53.90	4.22
	46	MWCNT-COOH	57.08	5.82

There were no differences among groups (chi-square, $P > 0.05$). *n* = number of embryos

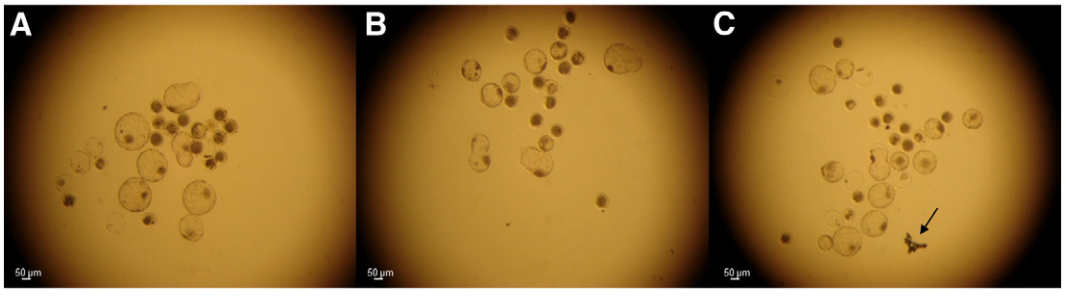

Figure 2. Photomicrographs of bovine embryos untreated **(A)**, treated with cotton cellulose nanofibers (CNF) **(B)** and carboxylated multiwalled carbon nanotubes (MWCNT-COOH) **(C)**. The black arrow indicates the MWCNT-COOH nanoparticle aggregates. Magnification ×40.

typical morphologies can be observed of the MWCNT-COOH with diameters that ranged from 10 to 50 nm and lengths that ranged from 5 to 30 μm (Figure 1, *B*). Zetasizer exhibited a Zeta potential of −6.98 mV for cotton CNF and −5.91 mV for MWCNT-COOH.

Embryonic development

There were no differences in the hatching rate ($P = 0.24$) and degeneration ($P = 0.17$) at 72 h of culture among the control, cotton CNF and MWCNT-COOH-exposed embryos (Table 2 and Figure 2).

Gene expression analysis

In embryos exposed to 0.2 μg mL^{-1} cotton CNF, only one gene *OCT4* (0.67 ± 0.22) was down-regulated ($P < 0.05$) compared with the control (Figure 3, *A*). In contrast, in embryos exposed at 0.2 μg mL-1 MWCNT-COOH showed relatively higher levels ($P < 0.05$) for the *HSP70.1* (2.06 ± 0.60), *PRDX1* (1.30 ± 0.46), *BAX* (1.66 ± 0.57), *HAD1* (1.39 ± 0.45) and *OCT4* (1.30 ± 0.40) transcripts when compared to the control group (Figure 3, *A*). The specificity of real time products PCR was checked by electrophoresis of real time PCR products on the agarose gel (Figure 3, *B*).

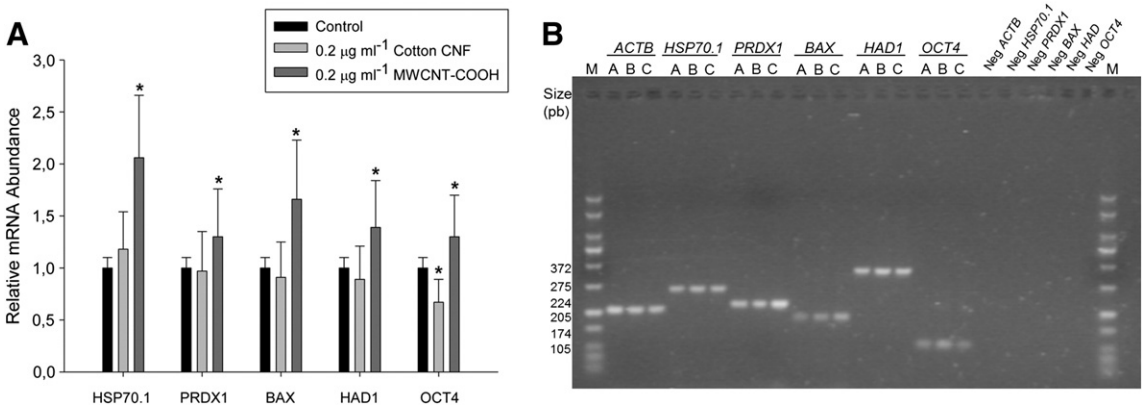

Figure 3. Quantitative gene expression analysis and confirmation of amplicon size of the genes. **(A)** Relative expression (mean ± SEM) of heat shock protein 70.1 (HSP70.1), peroxiredoxin 1 (PRDX1), BCL2-associated X protein (BAX), histone deacetylase 1 (HAD1) and octamer-binding transcription factor 4 (OCT4) transcripts in bovine embryos cultured without nanomaterials (control group), and with 0.2 μg mL^{-1} cotton cellulose nanofibers (CNF) or carboxylated multiwalled carbon nanotubes (MWCNT-COOH) for 72 h. Cotton CNF or MWCNT-COOH compared with the control group (relative expression = 1.00) ($P > 0.05$; mean ± SEM.) **(B)** Agarose gel electrophoresis showing specific real time PCR products of the expected size for each gene. "M" represents DNA size marker, "A" represents control group, "B" represents cotton CNF group, "C" represents MWCNT-COOH group and "Ne" represents negative control.

Table 3
Effect of cotton cellulose nanofibers (CNF) and multiwalled carbon nanotubes (MWCNT-COOH) (0.2 μg mL^{-1}) on blastocyst total cell number and apoptosis.

Groups	*n*	Total cell no.	No. of apoptotic cells	Apoptotic index
Control	19	130.88 ± 5.59	13.22 ± 1.05	10.28 ± 2.83
Cotton CNF	16	126.06 ± 6.93	14.43 ± 1.62	11.46 ± 3.57
MWCNT-COOH	16	127.81 ± 8.04	14.37 ± 1.99	10.28 ± 1.51

Data are the mean ± SEM. There were no differences among groups (ANOVA, $P > 0.05$). *n* = number of embryos.

Apoptosis analyses

There was no difference in total cell numbers ($P = 0.91$), and apoptotic cell index ($P = 0.82$) at 72 h of culture among the control and the cotton CNF or MWCNT-COOH-exposed embryos (Table 3, Figure 4).

Discussion

Interactions of cotton CNF and MWCNT-COOH with bovine embryos, were evaluated by the rates of embryonic development, gene expression and *in situ* apoptosis. The preimplantation embryonic period is characterized by distinct phases of morphological changes which include cleavage, compaction, expansion and hatching. Particularly, hatching is a critical event in the development, being related to the embryo's capacity to survive.[17] On the other hand, degeneration rates reflect changes in development resulting from arrest of cell division cycles[18] and subsequent cell death. Thus, the evaluation of these parameters is important when studying the influence of NMs on embryo development and survival. Cotton CNFs and MWCNT-COOHs have great application potential both in industry and biomedicine fields. However, the risks of maternal exposure during the embryonic period are poorly understood or unknown regarding cotton CNF and MWCNT-COOHs, respectively. Studies about toxicity of CNTs in embryos have been performed in aquatic species such as zebrafish[19,20] and few tests have been performed on mammalian species.[21–23] For instance, cotton CNFs' effects are not yet known for any stage of embryonic development of mammals. Bovine embryos are good models for the evaluation of the teratogenic potential of substances, because they are sensitive to toxicants and due to their morphological and biochemical similarities to human embryos[13,14] they can be used to evaluate the toxic effects of NM.

The preimplantation phase is possibly the most critical period of development due to the existence of cells that are still undifferentiated. Disturbances in this stage can cause failures in the implantation or lead to damage to cell division or differentiation processes which result in fetal tissues.[24] In this study, the cotton CNF and MWCNT-COOH did not change the hatching and degeneration rates. However, previous studies using another type of CNT (SWCNTs), at higher concentrations (30 μg mL^{-1}) and embryonic post-hatching stage, have shown fetal malformations and increased reactive oxygen species (ROS) production in mice.[25] More recently, Campagnolo et al (2013)[22] showed that functionalized SWCNTs might cause occasional teratogenic effects in mice. Non-functionalized MWCNT was fetotoxic to mice.[23] This inconsistency may be due to the chemical functionalization and/or type of the CNT. In the present study, we used the carboxylic functional group on the MWCNT surface. Previous studies have shown that CNT surface functionalization may alter the toxicity response.[26,27] To our knowledge, regarding the cotton CNF, there are no reports of embryotoxicity for these NMs. Previous work in our laboratory also showed that cotton CNF (0.02 to 100 μg mL^{-1}) exposure does not induce toxic effects in bovine fibroblasts.[15] Injuries in embryonic cells cause a higher proportion of unhatched or degenerated embryos.[13] Thus, in the present study, the maintenance of such rates suggests that cotton CNF and MWCNT-COOH, under the

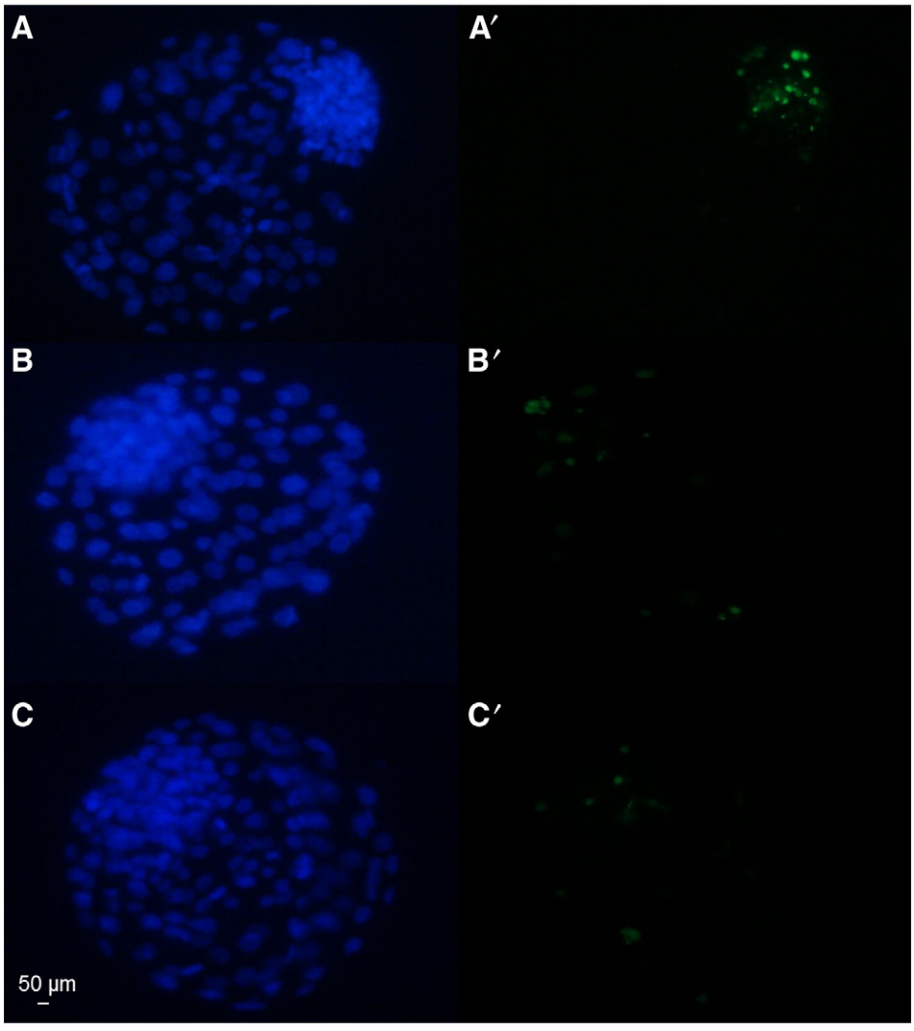

Figure 4. Representative images of TUNEL-labeled nucleic in bovine blastocysts cultured from the control group **(A, A′)** with 0.2 µg mL^{-1} cotton cellulose nanofibers (CNF) **(B, B′)** or carboxylated multiwalled carbon nanotubes (MWCNT-COOH) **(C, C′)** for 72 h. **(A), (B)** and **(C)** show the total number of cells (blue fluorescence) and **(A′)**, **(B′)** and **(C′)** show the number of apoptotic cells (green fluorescence). Optical microscopy fluorescence with ×100 magnification.

conditions tested, did not influence the kinetics of the development and survival of the bovine embryos produced *in vitro*.

To date, several studies have been conducted to better understand the mechanisms which might be involved in the NM toxicity. The most common cytotoxic effect is oxidative stress which causes damage to organelles or plasma membrane.[28] Because of their needle-like shapes, CNTs might be able to perforate cellular membrane and organelles.[29] Together, the shape, size and chemical composition are key components in the toxicity of NMs[28,30] and may be related to the gene expression changes observed in this study.

To further investigate possible molecular alterations induced by cotton CNF and MWCNT-COOH, we examined the expression of genes associated with response to stress (*HSP70.1* and *PRDX1*), apoptosis (*BAX*) and (*HAD1*, *OCT4*) by real time PCR analysis. *HSP70* overexpression may be considered as stress and cell death biomarkers. *HSP70s* act as molecular chaperones in remodeling proteins altered by stress, protecting cellular homeostasis.[31] Under normal physiological conditions, these proteins are expressed at low levels. However, ROS production, changes in temperature, heavy metals or other environmental stresses can increase intracellular levels of HSPs.[32] Okuda-Shimazaki et al (2010)[33] related an increase in *HSP70.1* expression due to cellular stress induced by aggregates or agglomerates of NM. In the present study, we also observed the formation of NM aggregates in embryo culture medium (Figure 2), and an increased expression of *HSP70.1* in embryos exposed to MWCNT-COOH (Figure 3, *A*). Zeta potential is a critical parameter, which determines nanoparticle stability or aggregation in dispersion. As a rule of thumb, absolute Zeta potential values above 30 mV provide good stability.[27] In the present study, Zeta potential values were −6.98 for cotton CNF and −5.91 mV for MWCNT-COOH demonstrating that NMs in culture medium are unstable solutions. However, *HSP70.1* gene expressions did not change in embryos exposed to cotton CNF (Figure 3, *A*). Similarly, the CNF aggregates were associated with lower toxicity.[1] A possible explanation for this event is that the aggregation or agglomeration of NM can affect the degree of uptake and toxicity based on the NM composition and the cell type.[34] Therefore, aggregation can trigger different toxic

responses which can be affected by many different NM, as well as by the biological model studied.

PRDX1 is a member of the *PRDX* family of antioxidant proteins involved in redox regulation of the cell. In adverse physiological conditions, such as ROS production increases, cells usually up-regulate the expression of *PRDX1*.[35] Previous studies have shown that oxidative stress conditions caused an increase in the level of *PRDX1* in embryos cultured *in vitro*.[36] In the present study, the *PRDX1* gene was up-regulated in embryos exposed to MWCNT-COOH (Figure 3, *A*). Oxidative stress has been implicated as the primary mechanism of cytotoxicity induced by CNT.[37] NMs are known to stimulate the cells' ability to produce toxic ROS due to their large surface area.[38] Therefore, the biological activity of MWCNT-COOHs due this large surface area may have generated a high pro-oxidant potential in the embryos used in this study. Probably, *PRDX1* up-regulation may represent a cellular response of embryos to minimize oxidative stress. Rostila et al (2012)[39] identified high plasma levels of the *PRDX1* proteins in human patients exposed to other fibers, like asbestos. These same authors suggested that *PRDX1* could be a biomarker for fiber exposure. Similarly, the *PRDX1* expression can be an indicator of oxidative stress in cells exposed to MWCNT-COOH.

Cell death is one of the consequences of oxidative stress stimulated by NM.[40] Cheng et al (2011)[41] observed ROS production and apoptosis in rat endothelial cells exposed to high levels of SWCNTs. In the present study, the abundance of transcripts for BAX was high in embryos exposed to MWCNT-COOH. This gene expresses a pro-apoptotic protein which induces cell death in response to apoptotic stimuli.[42] Similarly, the MWCNT exposure increased BAX levels in p53 knockout mice.[23] The same cellular response occurred after exposure of human liver cells to titanium dioxide nanoparticles[43] and A549 pulmonary cells to silver nanoparticles.[44] Therefore, BAX gene up-regulation appears to be a frequent event when cells are exposed to some types of NMs.

The *HAD1* proteins act on one of the main mechanisms of epigenetic regulation of the genes, promoting the deacetylation of histones. The deacetylation of histone promotes condensation of chromatin and repression of gene expression by leaving the transcription factor binding sites inaccessible. In the pre-implantation period, *HAD1* plays an important role in gene repression, regulating correct cell differentiation.[45] In *Xenopus* embryos, the inactivation of this gene has been directly correlated with birth defects.[46] In embryonic stem cells and trophoblastic cells, *HAD1* proteins bind to active genes.[47] Therefore, the up-regulation of this gene in embryos exposed to MWCNT-COOH may have a potential impact on the developing embryo. However, more studies are needed to understand the consequences of gene expression deregulation in embryos exposed to that NM.

OCT4 is a transcription factor whose expression is associated with an undifferentiated cell phenotype in the early mammalian embryo and is down-regulated when such cells differentiate.[48] In the present study, cotton CNF induced decreased expression of *OCT4*, while the MWCNT-COOH caused an increase in transcripts for this gene. A recent study found that the embryo development was not affected when depleted of maternal *OCT4* by the Cre-lox system.[49] In this study, the *OCT4* gene expression alteration did not affect embryonic development until the blastocyst stage (Figure 2, Table 2). However, additional studies are required to determine the possible consequences of *OCT4* gene expression alteration on later stages of mammalian embryonic development.

In order to examine the consequences of gene expression variation, we studied the effects of cotton CNF or MWCNT-COOH on the incidence of apoptosis in blastocysts. TUNEL assay is based on the activity of the terminal deoxynucleotidyl transferase TdT enzyme, which promotes the binding of nucleotides labeled with fluorescein at the 3′OH ends of DNA fragments which form during apoptosis.[50] The process of cell death in embryos has the function of eliminating the abnormal cells during the cell cycle (divisions) thus increasing the number of blastomeres. Apoptosis can occur due to the stress caused by *in vitro* culture system, but usually affects the embryos at low rates, and it is considered a normal developmental process. Therefore, both embryos produced *in vivo* and *in vitro* have blastomeres in the death process and the proportion of cells in this condition can achieve approximate values of 20% in the same embryo.[51] In this study, the apoptotic rates of the embryos did not exceed the values found in the literature, both in the control group (10.28 ± 2.83) and in those exposed to the cotton CNF (11.46 ± 3.57) or to MWCNT-COOHs (10.28 ± 1.51) (Table 3).

However, previous studies showed that the exposure of mouse embryonic stem cells to MWCNT could induce apoptosis by DNA damage.[52] In this case, they used higher MWCNT concentrations (100 μg mL^{-1}) compared to the present study (0.2 μg mL^{-1}) and the embryonic cells were in direct contact with the MWCNTs. Additionally, in the present study the embryonic cells were protected by the pellucid zone. This structure is formed by a thick layer of glycoproteins (10.5 μm) and has the function of protecting the oocyte and the embryo from chemical or physical injuries.[53] This inconsistency of results may also be due to the chemical functionalization of the MWCNT. In the present study, we used carboxylate MWCNT. Several studies have revealed that CNT surface functionalization can improve biocompatibility.[54,55]

From Figures 2 and 4, the cotton CNF did not have any negative impact on the embryo quality with regards to parameters at morphological and molecular level. Studies on toxic effects of cotton CNF are scarce for mammalian cells and nonexistent for mammalian embryos in the literature. Under the conditions tested in the present study, cotton CNF is more biocompatible in mammalian embryos when compared to MWCNT-COOH. Physicochemical characteristics of the cotton CNFs such as a biodegradability, flexibility and the chemical constitution[21] seem to be decisive in the toxic responses of organisms exposed to this NM.

In conclusion, our results add new insights into the potential biocompatibility of cotton CNF and MWCNT-COOH, which may help us to better understand the toxicity and potential biomedical application of these NMs. The gene expression was the only analyzed parameter affected by exposure of bovine embryos to cotton CNF and MWCNT-COOH. Real time PCR results showed that the MWCNT-COOH changed the abundance of transcripts relevant for the embryonic development and

homeostasis more intensely when compared with cotton CNF. On the other hand, cotton CNF and MWCNT-COOH did not induce any changes in viability indicators such as hatching rate, the total cell number, degeneration or apoptosis.

References

1. Lima R, Feitosa LO, Maruyama CR, Barga MA, Yamawaki PC, Vieira IJ, et al. Evaluation of the genotoxicity of cellulose nanofibers. *Int J Nanomedicine* 2012;**7**:3555-65, http://dx.doi.org/10.2147/IJN.S30596.
2. Lin N, Dufresne A. Surface chemistry, morphological analysis and properties of cellulose nanocrystals with gradiented sulfation degrees. *Nanoscale* 2014;**4**:5384-93, http://dx.doi.org/10.1039/c3nr06761k.
3. Monaco AM, Giugliano M. Carbon-based smart nanomaterials in biomedicine and neuroengineerin. *Beilstein J Nanotechnol* 2014;**5**:1849-63, http://dx.doi.org/10.3762/bjnano.5.196.
4. Meng L, Zhang X, Lu Q, Fei Z, Dyson PJ. Single walled carbone nanotubes as drug delivery vehicles: targeting doxorubicin to tumors. *Biomaterials* 2012;**33**:1689-98, http://dx.doi.org/10.1016/j.biomaterials.2011.11.004.
5. Mohanta V, Madras G, Patil S. Layer-by-layer assembled thin films and microcapsules of nanocrystalline cellulose for hydrophobic drug delivery. *ACS Appl Mater Interfaces* 2014;**6**:20093-101, http://dx.doi.org/10.1021/am505681e.
6. Zander NE, Dong H, Steele J, Grant JT. Metal cation cross-linked nanocellulose hydrogels as tissue engineering substrates. *ACS Appl Mater Interfaces* 2014;**6**:18502-10, http://dx.doi.org/10.1021/am506007z.
7. Newman P, Minett A, Ellis-Behnke R, Zreiqat H. Carbon nanotubes: their potential and pitfalls for bone tissue regeneration and engineering. *Nanomedicine* 2013;**8**:1139-58, http://dx.doi.org/10.1016/j.nano.2013.06.001.
8. Pourasl AH, Ahmadi MT, Rahmani M, Chin HC, Lim CS, Ismail R, et al. Analytical modeling of glucose biosensors based on carbon nanotubes. *Nanoscale Res Lett* 2014;**9**:33, http://dx.doi.org/10.1186/1556-276X-9-33.
9. Makhluf SB, Abu-Mukh R, Rubinstein S, Breitbart H, Gedanken A. Modified PVA-Fe3O4 nanoparticles as protein carriers into sperm cells. *Small* 2008;**4**:1453-8, http://dx.doi.org/10.1002/smll.200701308.
10. Chaudhury K, Babu KN, Singh AK, Das S, Kumar A, Seal S. Mitigation of endometriosis using regenerative cerium oxide nanoparticles. *Nanomedicine* 2013;**3**:439-48, http://dx.doi.org/10.1016/j.nano.2012.08.001.
11. Barkalina N, Jones C, Kashir J, Coote S, Huang X, Morrison R. Effects of mesoporous sílica nanoparticles upon the function of mammalian sperm *in vitro*. *Nanomedicine* 2014;**4**:859-70, http://dx.doi.org/10.1016/j.nano.2013.10.011.
12. Fubini B, Ghiazza M, Fenoglio I. Physico-chemical features of engineered nanoparticles relevant to their toxicity. *Nanotoxicology* 2010;**4**:347-63, http://dx.doi.org/10.3109/17435390.2010.509519.
13. Leidenfrost S, Boelhauve M, Reichenbach M, Güngör T, Reichenbach HD, Sinowatz F, et al. Cell arrest and cell death in mammalian preimplantation development: lessons from the bovine model. *PLoS One* 2011;**6**:e22121, http://dx.doi.org/10.1371/journal.pone.0022121.
14. Baumann CG, Morris DG, Sreenan JM, Leese HJ. The quiet embryo hypothesis: molecular characteristics favoring viability. *Mol Reprod Dev* 2007;**74**:1345-53, http://dx.doi.org/10.1002/mrd.20604.
15. Pereira MM, Raposo NR, Brayner R, Teixeira EM, Oliveira V, Quintão CCR, et al. Cytotoxicity and expression of genes involved in the cellular stress response and apoptosis in mammalian fibroblast exposed to cotton cellulose nanofibers. *Nanotechnology* 2013;**24**:075103, http://dx.doi.org/10.1088/0957-4484/24/7/075103.
16. Pfaffl MW, Horgan GW, Dempfle L. Relative Expression Software Tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 2002;**30**:26-36, http://dx.doi.org/10.1093/nar/30.9.e36.
17. Trigal B, Muñoz M, Gómez E, Caamaño JN, Martin D, Carrocera S, et al. Cell counts and survival to vitrification of bovine *in vitro* produced blastocysts subjected to sublethal high hydrostatic pressure. *Reprod Domest Anim* 2013;**48**:200-6, http://dx.doi.org/10.1111/j.1439-0531.2012.02131.x.
18. Ma W, Yang X, Liang X. Obesity does not aggravate vitrification injury in mouse embryos: a prospective study. *Reprod Biol Endocrinol* 2012;**10**:68, http://dx.doi.org/10.1186/1477-7827-10-68.
19. Cheng J, Cheng SH. Influence of carbon nanotube length on toxicity to zebrafish embryos. *Int J Nanomedicine* 2012;**7**:3731-9, http://dx.doi.org/10.2147/IJN.S30459.
20. Liu XT, Mu XY, Wu XL, Meng LX, Guan WB, Ma YQ, et al. Toxicity of multi-walled carbon nanotubes, grapheme oxide, and reduced grapheme oxide to zebrafish embryos. *Biomed Environ Sci* 2014;**27**:676-83, http://dx.doi.org/10.3967/bes2014.103.
21. Lim JH, Kim SH, Shin IS, Park NH, Moon C, Kang SS, et al. Maternal exposure to multi-wall carbono nanotubes does not induce embryo-fetal developmental toxicity in rats. *Birth Defects Res B Dev Reprod Toxicol* 2011;**92**:69-76, http://dx.doi.org/10.5620/eht.2011.26.e2011006.
22. Campagnolo L, Massimiani M, Palmieri G, Bernardini R, Sacchetti C, Bergamaschi A, et al. Biodistribution and toxicity of pegylated single wall carbon nanotubes in pregnant mice. *Part Fibre Toxicol* 2013;**10**:21, http://dx.doi.org/10.1186/1743-8977-10-21.
23. Huang X, Zhang F, Sun X, Choi KY, Niu G, Zhang G, et al. The genotype-dependent influence of functionalized multiwalled carbon nanotubes on fetal development. *Biomaterials* 2014;**35**:856-65, http://dx.doi.org/10.1016/j.biomaterials.2013.10.027.
24. Zalgevičienė L, Kulvietis V, Bulotienė D, Didžiapetrienė J, Rotomskis R. The effect of nanoparticles in rats during critical periods of pregnancy. *Medicina (Kaunas)* 2012;**48**:256-64.
25. Pietroiusti A, Massimiani M, Fenoglio I, Colonna M, Valentini F, Palleschi G, et al. Low doses of pristine and oxidized single-wall carbon nanotubes affect mammalian embryonic development. *ACS Nano* 2011;**5**:4624-33, http://dx.doi.org/10.1021/nn200372g.
26. Vardharajula S, Ali SZ, Tiwari PM, Eroğlu E, Vig K, Dennis VA, et al. Functionalized carbon nanotubes: biomedical applications. *Int J Nanomedicine* 2012;**7**:5361-74, http://dx.doi.org/10.2147/IJN.S35832.
27. Jiang Y, Zhang H, Wang Y, Chen M, Ye S, Hou Z, et al. Modulation of apoptotic pathways of macrophages by surface-functionalized multi-walled carbon nanotubes. *PLoS One* 2013;**8**:65756, http://dx.doi.org/10.1371/journal.pone.0065756.
28. Doshi N, Mitragotri S. Needle-shaped polymeric particles induce transient disruption of cell membranes. *J R Soc Interface* 2010;**7**:403-10, http://dx.doi.org/10.1016/j.tiv.2011.09.017.
29. Kostarelos K. The long and short of carbon nanotube toxicity. *Nat Biotechnol* 2008;**26**:774-6, http://dx.doi.org/10.1038/nbt0708-774.
30. Yamashita K, Yoshioka Y, Higashisaka K, Morishita Y, Yoshida T, Fujimura M, et al. Carbon nanotubes elicit DNA damage and inflammatory response relative to their size and shape. *Inflammation* 2010;**33**:276-80, http://dx.doi.org/10.1007/s10753-010-9182-7.
31. Cicchetti R, Divizia M, Valentini F, Argentin G. Effects of single-wall carbon nanotubes in human cells of the oral cavity: geno-cytotoxic risk. *Toxicol In Vitro* 2011;**25**:1811-9, http://dx.doi.org/10.1016/j.tiv.2011.09.017.
32. Bianchi A, Moulin D, Hupont S, Koufany M, Netter P, Reboul P, et al. Oxidative stress-induced expression of HSP70 contributes to the inhibitory effect of 15d-PGJ2 on inducible prostaglandin pathway in chondrocytes. *Free Radic Biol Med* 2014;**76**:114-26, http://dx.doi.org/10.1016/j.freeradbiomed.
33. Okuda-Shimazaki J, Takaku S, Kanehira K, Sonezaki S, Taniguchi A. Effects of titanium dioxide nanoparticle aggregate size on gene expression. *Int J Mol Sci* 2010;**11**:2383-92, http://dx.doi.org/10.3390/ijms11062383.
34. Albanese A, Chan WC. Effect of gold nanoparticle aggregation on cell uptake and toxicity. *ACS Nano* 2011;**5**:5478-89, http://dx.doi.org/10.1021/nn2007496.

35. Li B, Ishii T, Tan CP, Soh JW, Goff SP. Pathways of induction of peroxiredoxin I expression in osteoblasts: roles of p38 mitogen-activated protein kinase and protein kinase C. *J Biol Chem* 2002;**277**:12418-22, http://dx.doi.org/10.1074/jbc.M111443200.
36. Pereira MM, Machado MA, Costa FQ, Serapiao RV, Viana JH, et al. Effect of oxygen tension and serum during IVM on developmental competence of bovine oocytes. *Reprod Fertil Dev* 2010;**22**:1074-82, http://dx.doi.org/10.1071/RD10007.
37. Alarifi S, Ali D. Mechanisms of multi-walled carbon nanotubes-induced oxidative stress and genotoxicity in mouse fibroblast cells. *Int J Toxicol* 2015;**34**:258-65, http://dx.doi.org/10.1177/1091581815584799.
38. Pujalté I, Passagne I, Brouillaud B, Tréguer M, Durand E, Ohayon-Courtès C, et al. Cytotoxicity and oxidative stress induced by different metallic nanoparticles on human kidney cells. *Part Fibre Toxicol* 2011;**8**:10, http://dx.doi.org/10.1186/1743-8977-8-10.
39. Rostila A, Puustinen A, Toljamo T, Vuopala K, Lindström I, Nyman TA, et al. Peroxiredoxins and tropomyosins as plasma biomarkers for lung cancer and asbestos exposure. *Lung Cancer* 2012;**77**:450-9, http://dx.doi.org/10.1016/j.lungcan.2012.03.024.
40. Ramkumar KM, Manjula C, Gnanakumar G, Kanjwal MA, Sekar TV, Paulmurugan R, et al. Oxidative stress-mediated cytotoxicity and apoptosis induction by TiO2 nanofibers in HeLa cells. *Eur J Pharm Biopharm* 2012;**81**:324-33, http://dx.doi.org/10.1016/j.ejpb.2012.02.013.
41. Cheng WW, Lin ZQ, Wei BF, Zeng Q, Han B, Wei CX, et al. Single-walled carbon nanotube induction of rat aortic endothelial cell apoptosis: reactive oxygen species are involved in the mitochondrial pathway. *Int J Biochem Cell Biol* 2011;**43**:564-72, http://dx.doi.org/10.1016/j.biocel.2010.12.013.
42. Van Delft MF, Huang DC. How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell Res* 2006;**16**:203-13, http://dx.doi.org/10.1038/sj.cr.7310028.
43. Yoo KC, Yoon CH, Kwon D, Hyun KH, Woo SJ, Kim RK, et al. Titanium dioxide induces apoptotic cell death through reactive oxygen species-mediated Fas upregulation and Bax activation. *Int J Nanomedicine* 2012;**7**:1203-14, http://dx.doi.org/10.2147/IJN.S28647.
44. Lee YS, Kim DW, Lee YH, Oh JH, Yoon S, Choi MS, et al. Silver nanoparticles induce apoptosis and G2/M arrest via PKCζ-dependent signaling in A549 lung cells. *Arch Toxicol* 2011;**85**:1529-40, http://dx.doi.org/10.1007/s00204-011-0714-1.
45. Wang Z. Histone deacetylase 1 down-regulation on developmental capability and histone acetylation in bovine oocytes and parthenogenetic embryos. *Reprod Domest Anim* 2011;**46**:1022-8, http://dx.doi.org/10.1111/j.1439-0531.2011.01778.x.
46. Carneiro K, Donnet C, Rejtar T, Karger BL, Barisone GA, Díaz E, et al. Histone deacetylase activity is necessary for left right patterning during vertebrate development. *BMC Dev Biol* 2011;**11**:29, http://dx.doi.org/10.1186/1471-213X-11-29.
47. Kidder BL, Palmer S. HDAC1 regulates pluripotency and lineage specific transcriptional networks in embryonic and trophoblast stem cells. *Nucleic Acids Res* 2012;**40**:2925-39, http://dx.doi.org/10.1093/nar/gkr1151.
48. Frum T, Halbisen MA, Wang C, Amiri H, Robson P, Ralston A. Oct4 cell-autonomously promotes primitive endoderm development in the mouse blastocyst. *Dev Cell* 2013;**25**:610-22, http://dx.doi.org/10.1016/j.devcel.2013.05.004.
49. Le Bin GC, Munoz-Descalzo S, Kurowski A, Leitch H, Lou X, Mansfield W, et al. Oct4 is required for lineage priming in the developing inner cell mass of the mouse blastocyst. *Development* 2014;**141**:1001-10, http://dx.doi.org/10.1242/dev.096875.
50. Kyrylkova K, Kyryachenko S, Leid M, Kioussi C. Detection of apoptosis by TUNEL assay. *Methods Mol Biol* 2012;**887**:41-7, http://dx.doi.org/10.1007/978-1-61779-860-3_5.
51. Neuber E. Analysis of DNA fragmentation of *in vitro* cultured bovine blastocysts using TUNEL. *Theriogenology* 2002;**57**:2193-202, http://dx.doi.org/10.1016/S0093-691X(02)00901-9.
52. Zhu L, Chang DW, Dai L, Hong Y. DNA damage induced by multiwalled carbon nanotubes in mouse embryonic stem cells. *Nano Lett* 2007;**7**:3592-7, http://dx.doi.org/10.1021/nl071303v.
53. Gupta SK, Bansal P, Ganguly A, Bhandari B, Chakrabarti K. Human zona pellucida glycoproteins: functional relevance during fertilization. *J Reprod Immunol* 2009;**83**:50-5, http://dx.doi.org/10.1016/j.jri.2009.07.008.
54. Charbgoo F, Behmanesh M, Nikkhah M. Enhanced reduction of single-wall carbon nanotube cytotoxicity *in vitro*: applying a novel method of arginine functionalization. *Biotechnol Appl Biochem* 2015, http://dx.doi.org/10.1002/bab.1311.
55. Mata D, Amaral M, Fernandes AJ, Colaço B, Gama A, Paiva MC, et al. Diels-Alder functionalized carbon nanotubes for bone tissue engineering: *in vitro*/*in vivo* biocompatibility and biodegradability. *Nanoscale* 2015;**7**:9238-51, http://dx.doi.org/10.1039/c5nr01829c.